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by Matthew D. Servinsky, Patrick C. Allen, Chen-Yu Tsao, Christopher M. Byrd, Christian J. Sund, and William E. Bentley

ARL-RP-0402 **September 2012**

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14. ABSTRACT
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Abstract

Microbially Induced Corrosion (MIC) occurs at metal surfaces and is associated with microorganisms and their metabolic activities. These microbes can coexist as biofilms, growing as synergistic communities (consortia) that are able to affect electrochemical processes, both cathodic and anodic, often through co-operative metabolism. Recent research has revealed the role of "quorum sensing" molecules in control of microbial activities such as biofilm formation. In this paper, we propose the detection of quorum sensing molecules as a means of detecting bacterial contamination prior to the onset on biofilm formation. Further we outline the development of an *E. coli* cell based sensor for detection of the quorum sensing molecule Autoinducer-2 (AI-2).

Keywords: microbiologically induced corrosion, quorum sensing, Autoinducer-2 (AI-2), sensor cell, biofilm

1. Introduction

The study of microorganisms acting together as a biofilm is a flourishing area of research with medical, environmental, and industrial implications. Counter intuitively, these biofilms can exist in petroleum transport, storage, and delivery systems, causing corrosion and other mechanical failures resulting in increased operational and maintenance costs. This process, known as microbially induced corrosion (MIC), is an electrochemical process in which microorganisms initiate or enhance a corrosion reaction at a metal surface. MIC occurs in virtually all industries, including paper and pulp, sugar, dentistry, and, of particular interest to the Army, the shipping, gas, and petroleum industries [1]. An estimated 40% of internal pipeline corrosion in the petroleum industry is caused by microbes [2]. Moisture and other inorganic contaminants stratify at the bottom of fuel storage containers and pipelines. As a result, microbes are able to colonize the aqueous phase and metabolize the hydrocarbons that accumulate at the interface between the aqueous and organic phases. Detection of organisms causing MIC is problematic for a number of reasons. Firstly, some organisms found forming biofilms in fuel lines and storage containers are anaerobic, making culture based detection costly and prone to false negatives. Additionally, biofilms are localized, cohesive structures that do not readily release cells at a significant rate so detection strategies relying on organism growth or PCR amplification of DNA may produce false negatives. Numerous bacterial contaminants of logistics fuels have been identified, but there is currently no sensor system to determine if a fuel system contains these bacteria or if these organisms are causing MIC. Therefore, a method for identifying biofilm formation in systems not amenable to traditional culturing techniques will allow for operators to detect MIC and identify systems that may potentially fail due to this costly and understudied problem.

Bacteria, once thought to be autonomously operating units, are now known to utilize an intricate communication system for sensing and interpreting environmental cues that coordinate population-based behavior. One such system is quorum sensing (QS), and it involves the production, release, and detection of small chemical signaling molecules known as autoinducers (Figure 1). As the concentration of autoinducer increases, a threshold is achieved, stimulating a signal transduction cascade resulting in a coordinated change in the cellular processes of the population of organisms [3, 4]. QS has been found to regulate such diverse cellular processes as bioluminescence [5], virulence [6], biofilm formation [7], cell division, motility, metabolism, and recombinant protein production [8-10]. While there are many species-specific autoinducers, one type of signal molecule, autoinducer-2 (AI-2), has recently been linked to interspecies

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communication, indicating its potential as a 'universal' signal for QS circuitry among multiple bacterial species and has an instrumental role in coordinating biofilm formation [11]. In a recent study characterizing bacterial consortia isolated from petroleum-product-transporting pipelines, the distribution of species was found to be *B. cereus* ACE4 (30%), *S. marcescens* ACE2 (10%), and 10% of each species of *B. subtilis* AR12, *P. aeruginosa* AI1, *K. oxytoca* ACP, *P. stutzeri* AP2, *B. litoralis* AN1, and *Bacillus* sp. AN5 [1]. Of these, *B. cereus*, *S. marcescens*, and *B. subtilis* have been determined to produce AI-2 [12-14].

The presence of AI-2 is currently detected through a bioassay which has a number of limitations. The assay utilizes the reporter strain *Vibrio harveyi* BB170, a mutant strain of *V. harveyi* which does not produce AI-2, which produces bioluminescense in response to exogenously added AI-2 (15). A drawback to this strain is that the cells eventually emit bioluminescence in the absence of AI-2 once they have produced sufficient amounts of a second quorum sensing molecule, Autoinducer-1 (AI-I). The assay is performed by diluting an overnight culture of strain BB170 to reduce the AI-1 concentration prior to incubation with the sample. This requires that the assay be measured frequently to determine whether bioluminescence is induced from AI-2 in the test sample or from accumulation of endogenously produced AI-1 by comparison to the negative control. Another limitation is that the assay can be severely influenced from a variety of environmental conditions and has been shown to lack reproducibility, with large discrepancies reported between replicate experiments. Lastly, the assay is qualitative and not quantitative. [16-18].

The objective of the proposed research is to develop a method for detecting bacterial contamination of fuel vessels by developing a more robust AI-2 biosensor. There are several advantages of using QS molecules to detect bacterial contamination over direct culturing techniques or PCR based assays. Organisms growing in fuel vessels accumulate at the fuel water interface and are therefore not easily sampled, making culture based methods unreliable. Culture based methods also risk false positive results from environmental contamination. A QS based sensor would alleviate the need to isolate organisms from locations where direct sampling of the bacteria is not possible because the detection would be based on a diffusible molecule. PCR based assays are very sensitive for bacterial identification and can theoretically detect as few as one genome, but they do not give any information on the viability or metabolic activity of the cells; dead, metabolically inactive, and rapidly growing cells are indistinguishable. In contrast, cells must be actively metabolizing to produce autoinducer molecules, so a QS based sensor should only detect viable, metabolically active cells, which is a favorable property for detection of MIC because metabolic activity contributes to MIC.

The Bentley lab at the University of Maryland College Park recently developed a gene expression system that is controlled by the QS molecule AI-2. The cells used in this system do not produce AI-2 due to a mutation in the AI-2 synthesis gene *luxS,* but they have been engineered to respond to exogenously added AI-2. The cells contain the remainder of the native QS pathway, and the response to AI-2 is controlled by the binding of phosphorylated AI-2 to the QS repressor LsrR. The cells contain two plasmids which, when combined, propagate a fluorescent signal in response to the binding of phosphorylated AI-2 to LsrR, as shown in figure 1. Experiments in the Bentley lab showed that these cells produce a fluorescent protein in response to AI-2, and this system is the basis for the reporter system developed in this work [19].

Analysis of the QS pathway of *E. coli* showed that there were several places where the pathway could be engineered to respond to lower levels of AI-2 and increase the sensitivity of the reporter system (figure 1). In this study, we used genetic engineering to generate three modified versions of the sensor strain to increase internal levels of phosphorylated AI-2 and therefore increase the sensitivity of the AI-2 sensor. The first strategy was to increase the uptake of AI-2 by the cells through increased production of the AI-2 importer LsrACDB. The second strategy was to increase the production of LsrK, which is responsible for phosphorylation of AI-2. Phosphorylated AI-2 is the active form of the molecule and is also incapable of diffusing out of the cell, unlike unphosphorylated AI-2. The final strategy was to decrease AI-2 degradation by making deletions in the genes *lsrF* and *lsrG*, which encode proteins responsible for AI-2 degradation.

2. Materials and methods

2.1 Bacterial growth conditions and plasmid construction.

Plasmids and strains used in this study are listed in Table1. Strains were maintained and plasmids were manipulated according to standard procedures and previous reports [20]. Oligonucleotides were from Integrated DNA Technologies (Coralville, IA) and are listed in Table 2. DNA sequencing was performed by Genewiz (South Plainfield, NJ). All plasmids generated in this study were derived from pBAD28. Elimination of the ampicillin resistance capacity of pBad28 was achieved through deletion of a large portion of the ampicillin resistance gene by digestion with ApaLI and FspI, generating plasmid pBad28Δ*amp*. The *E. coli lsrK* and *lsrACDB* genes were amplified from strain W3110 genomic DNA using primer pairs (*lsrK* For / *lsrK* Rev) and (*lsrACDB* For / *lsrACDB* Rev) respectively. Each gene was individually cloned into pBad28Δ*amp* using the SacI and SalI restriction sites in the multiple cloning region, generating plasmids pBad28Δ*amplsrK* and pBadΔ*amplsrACDB* respectively.

2.2 Chromosomal deletions of *lsrFG* and *luxS*.

The one-step replacement method described by Datsenko and Wanner [21] was used to construct a deletion in *E. coli* W3110. We used the phage_ Red recombination system to replace *lsrFG* with the *lsrFG*::*cat* PCR *cat* cassette. The *cat* cassette was produced by PCR amplifying pKD3 using Vent DNA polymerase (New England Biolabs, MA) with primers lsrFGHP3 and lsrFGHP2 (Table 1). The blunt end PCR product was introduced by electroporation into *E. coli* W3110 containing plasmid pKD46, which expresses the Red recombinase, and was cured later by growth at 37°C. Recombinants were selected on LB broth supplemented with chloramphenicol, resulting in the strain W3110 (Δ*lsrFG*::*cat*). In order to remove the antibiotic resistance gene, the plasmid 707FLPe (tet^r) (Gene Bridges, Heidelberg, German) was chemically transferred into W3110 (Δ*lsrFG*::*cat*) using the Z-Competent™ *E. coli* Transformation Kit (Zymo Research, CA, USA). The expression of the plasmid mediated the excision of the antibiotic resistance gene, and the product strain was named W3110Δ*lsrFG*. Deletion of *luxS* from W3110Δ*lsrFG* to make the *lsrFG* and *luxS* double knockout strain was conducted similarly by PCR amplification of pKD3 with primers *luxS*HP1 and *luxS*HP2 (Table 2) [22], and the excision of the antibiotic resistance gene was similar to that described above. The product strain, W3110 ($lsrFG^-$, $luxS^-$), was named W3110Δ*lsrFG*Δ*luxS*. Deletion of *luxS* from W3110 was performed in the same manner, generating a strain named W3110Δ*luxS*. The primers used for PCR verification of gene deletions are listed in Table 2.

2.3 Transformation of cells.

Electrocompetent cells were created from overnight cultures diluted 1:50 into fresh LB broth with appropriate antibiotics. Cells were incubated in a shaking incubator at 37°C. Upon reaching mid-log phase, the cells were washed twice with ice-cold ultrapure water and twice with ice-cold 10% glycerol. 1 µL of DNA and 35 µL of cells were combined in a 1 mm electroporation cuvette. Electroporation was performed using a BioRad MicroPulser on the EC-1 setting. The cells were recovered for one hour before plating on LB agar with appropriate antibiotics [20].

2.4 Western Blot of LsrK and LsrACDB.

Overnight cultures were diluted 1:50 into fresh LB broth with antibiotics and allowed to grow in a shaking incubator at 37°C until an OD_{600} = 0.5 was reached. The culture was then split. One culture was a control and the other was induced with 0.2% arabinose. Both were grown for 3 hours. Samples were boiled for 1 minute in SDS-Page Buffer and loaded into a 10% Tris-HCl gel (Bio-Rad). The proteins were then transferred using an iBlot Dry Blotting System (Invitrogen). Western Blots were performed using the Western Breeze kit (Invitrogen), using approximately 2µg/mL if of Anti-LsrK

or Anti-LsrB as the primary antibody. The blots were imaged using a VersaDoc (Bio-Rad). Rabbit affinity purified antibodies for LsrK and LsrB were generated using synthesized peptides (Anti-LsrK: CVRWERTHTPDPEKH), (Anti-LsrB: CTWDSDTKPECRSYY) by GenScript.

Table 1 Bacterial strains and plasmids

Strain/plasmid	Relevant genotype and property	Source or reference
E. coli strains		
W3110	K12 strain, wild type, λ^-, F^-, IN(*rrnD-rrnE*)1, *rph-1*s	Genetic Stock Center Yale University, New Haven, CT
W3110 (Δ*lsrFG::cat*)	W3110 *luxS::cat*, W3110 *luxS* knockout strain with Chloramphenicol resistance	This study
W3110Δ*luxS*	W3110-derived *luxS* knockout strain	This study
W3110 (Δ*lsrFG::cat*)	W3110 *lsrFG::cat*, W3110 *lsrFG* knockout strain with Chloramphenicol resistance	This study
W3110Δ*lsrFG*	W3110-derived *lsrFG* knockout strain	This study
W3110Δ*lsrFG* (Δ*lsrFG::cat*)	W3110ΔlsrFG *luxS::cat*, W3110ΔlsrFG and *luxS* knockout strain with Chloramphenicol resistance	This study
W3110Δ*lsrFG*Δ*luxS*	W3110-derived *lsrFG* and *luxS* knockout strain	This study
Plasmids		
pET200/D-TOPO	Cloning vector, containing *T7* promoter, Km^r	Invitrogen
pBad28	Expression vector with Arabinose promoter, Ap^r, Cm^r	ATCC
pBad28Δ*amp*	pBad28 with ampicillin resistance gene removed	This study
pBad28Δ*amp*/*lsrK*	pBad28Δamp with *lsrK* cloned behind arabinose promoter	This study
pBad28Δ*amp*/*lsrACDB*	pBad28Δ*amp* with *lsrK* cloned behind arabinose promoter	This study
pCT6	pFZY1 derivative, containing *lsrR* and *lsrR* promoter region fused with *T7RPol*, Ap^r	[Tsao et al., 2010]
pET200/*dsRed*	pET200 derivative, containing *dsReduv*, Km^r	[Tsao et al., 2010]
pTrcHis-LuxS	pTrcHisC derivative, containing *luxS* from W3110, Ap^r	[Barrios et al., 2006]
pTrcHis-Pfs	pTrcHisC derivative, containing *pfs* from W3110, Ap^r	[Barrios et al., 2006]

Table 2. Primers

Primer name	Sequence	Relevant description
*lsrK*_For	TATTGAGCTCATGGCTCGACTCTTTACCCTTTC	Upstream Primer for cloning *lsrK* into pBad28Δ*amp*
*lsrK*_Rev	AACAGTCGACCTATAACCCAGGCGCTTTCC	Downstream Primer for cloning *lsrK* into pBad28Δ*amp*
*lsrACDB*_For	ATAGAGCTCATGCAAACGAGTGATACCCGCGCGTTAC	Upstream Primer for cloning *lsrACDB* into pBad28Δ*amp*
*lsrACDB*_Rev	TTATGTCGACTCAGAAATCGTATTTGCCGATATTCTCTTTGTTG	Downstream Primer for cloning *lsrACDB* into pBad28Δ*amp*
*lsrF*GHP3	ATCGGCAAATACGATTTCTGATGTGCATTACTTAACCGGAGTAAGTTATGGTGTAGGCTGGAGCTGCTTC	Primer for deletion of *lsrFG* gene
*lsrF*GHP2	GCTTGAAGCTTATAATTTCCCCCGTTCAGTTTTGAGATTCCAGTTTCGCGACACAGGTTTTGTAGTGGGGCGTGCATATGAATATCCTCCTTAG	Primer for deletion of *lsrFG* gene (Wang et al., 2005)
*luxS*HP1	ATGCCGTTGTTAGATAGCTTCACAGTCGATCATACCCGGAGTGTAGGCTGGAGCTGCTTC	Primer for deletion of *luxS* gene (Wang et al., 2005)
*luxS*HP2	CTAGATGTGCAGTTCCTGCAACTTCTCTTTCGGCAGTGCCCATATGAATATCCTCCTTAG	Primer for deletion of *luxS* gene
C1	TTATACGCAAGGCGACAAGG	Checking primer for Cm^r recombination
C2	GATCTTCCGTCACAGGTAGG	Checking primer for Cm^r recombination [Datsenko and Wanner, 2000]
CK*lsrF*1	GCGCGTGATATTCAACAAAGAG	Checking primer for *lsrFG* gene deletion [Datsenko and Wanner, 2000]
CK*lsrG*1	CACGGCATCAAACCATTGAAC	Checking primer for *lsrFG* gene deletion

3. Results

3.1 Generation of the phase I sensor

To generate the initial sensor strain, *luxS* was deleted from the wild type *E. coli* strain W3110. Plasmids pCT6 and pET200/dsRed [19] were sequentially transformed into the W3110 *luxS* deletion mutant, W3110Δ*luxS*, and grown in LB media with appropriate antibiotics. Plasmid pCT6 senses and responds to the presence of phosphorylated AI-2 as it has the *lsr* promoter controlling expression of T7 RNA polymerase in one direction and expression of LsrR in the other. When LsrR is bound by phosphorylated AI-2, the Lsr promoter is derepressed, initiating expression of the T7 RNA polymerase which, in turn, activates expression of dsRed from plasmid pET200/*dsRed*. Since the sensor cell line is *luxS* deficient and does not make AI-2, it should only produce red fluorescent protein in response to exogenous AI-2.

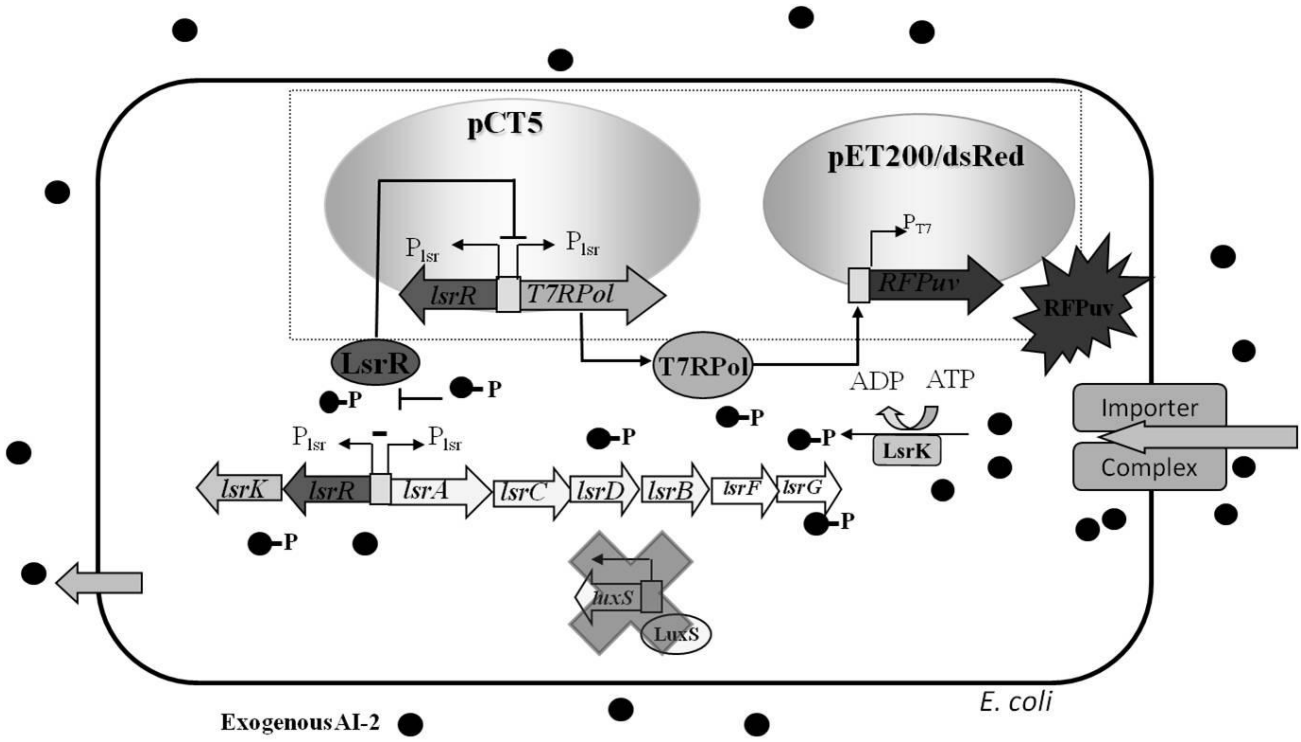

Figure 1. Schematic of the sensor cell. Plasmid pCT6 responds to phospho-AI-2 due to the presence of the bi-directional *lsr* promoter controlling expression T7 RNA polymerase. When LsrR is bound by phosphorylated AI-2, the Lsr promoter is derepressed, initiating expression of the T7 RNA polymerase which, in turn, activates expression of DsRed from plasmid pET200/*dsRed*. These plasmids were introduced into an *E. coli* cell line, W3110Δ*luxS,* which contains a deletion mutation in the *luxS* gene. Therefore, the cells do not synthesize AI-2; they should only fluoresce red in the presence of exogenous AI-2.

3.2 Engineering the Phase I sensor for enhanced sensitivity

Enhancing the sensor's sensitivity by increasing the expression of lsrACDB and lsrK required the maintenance of a third plasmid in the cells. This required finding a third plasmid with an origin of replication that would be compatible with pCT6 (oriF derived origin) and pET200/dsRed (pBR322 derived origin). Therefore, a novel plasmid was generated from pBad28 (p15a derived origin) with the ampicillin resistance gene removed. The resulting plasmid, named pBad28Δ*amp,* allowed for the selection of cells harboring all three plasmids in media containing chloramphenicol, kanamycin, and ampicillin. To increase the expression of the AI-2 importer, lsrACDB were cloned into pBad28Δ*amp* downstream of the arabinose promoter to generate pBad28Δ*amplsrACDB*. Expression of the AI-2 kinase (LsrK), which traps and activates AI-2 inside the cell, was increased by cloning lsrK downstream of the arabinose promoter in pBad28Δ*amp* to form pBad28Δ*amplsrK*.

Enhancing the sensor's sensitivity by eliminating the enzymes which degrade phosphorylated AI-2 from the Phase I sensor was not possible by deleting genes *lsrFG* directly from the Phase I sensor strain. The reason for this is not clear, but no viable cells were recovered after several attempts. Therefore *lsrFG* genes were deleted from the wild type *E. coli* strain W3110, creating the strain W3110Δ*lsrFG*. The *luxS* gene from strain W3110Δ*lsrFG* was subsequently deleted, creating strain W3110Δ*lsrFG*Δ*LuxS*.

3.3 Expression of LsrK and LsrACDB

To determine if plasmids pBad28Δ*amplsrK* and pBad28Δ*amplsrACDB* produce LsrK and LsrACDB respectively, Western Blots were performed. A band appeared in the sensor cells containing pBad28Δ*amp*/*lsrK* induced with arabinose (figure 2, lane 1) that was not present in the uninduced control (figure 2, lane 2). The band migrated slightly slower than the molecular weight standard of 50 kDa, which correlates well with the predicted molecular weight of LsrK, which is 57.5 kDa. No bands were detected in any lanes when blotting with anti-LsrB. We have yet to determine if this is a result of sample preparation or the integrity of the antibody.

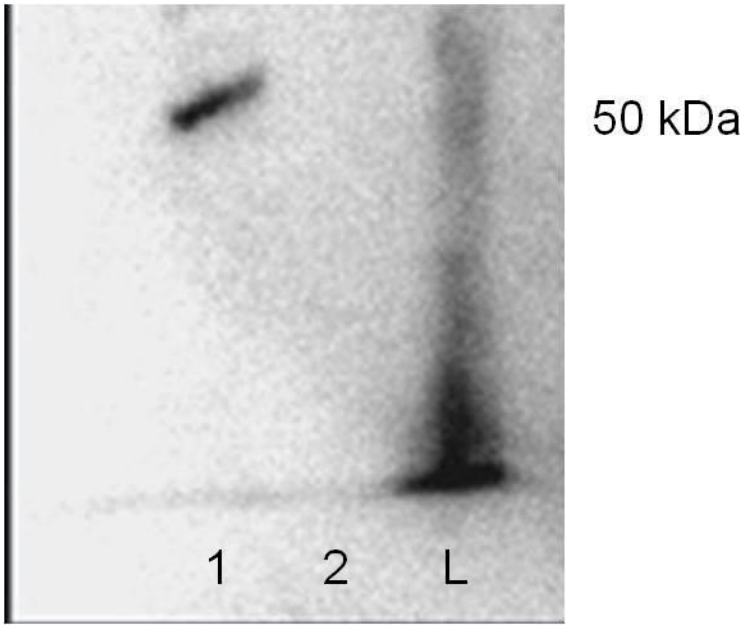

Figure 2. Western blot of the phase I sensor strain blotted with anti-LsrK. Lane 1 is the phase I sensor containing the plasmid pBad28Δamp/lsrK induced for 3 hours with 0.2% arabinose. Lane 2 is the phase I sensor containing the plasmid pBad28Δamp/lsrK mock induced for 3 hours.

4. Discussion

Microbial induced corrosion occurs in virtually all industries, ranging from food and medical to gas and petroleum. MIC is a well documented phenomenon which causes deterioration of logistic fuel pipelines and storage tanks. Corrosion affects the operation and maintenance costs of the pipelines, and many fuel pipelines face severe corrosion and microfouling problems. This poses serious concerns for the Army. If biologically derived fuels, such as biodiesel and bioethanol, become more prevalent, issues stemming from MIC will likely increase. The research presented here outlines the development of an *E. coli* cell based sensor which detects the quorum sensing molecule AI-2. As quorum sensing molecules accumulate prior to biofilm formation, the sensor has the potential for use in early detection of contamination in fuel storage vessels and pipelines.

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